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Distinct *delta* and *jagged* genes control sequential segregation of pancreatic cell types from precursor pools in zebrafish

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Abstract

The different cell types of the vertebrate pancreas arise asynchronously during organogenesis. Beta-cells producing insulin, alpha-cells producing glucagon, and exocrine cells secreting digestive enzymes differentiate sequentially from a common primordium. Notch signaling has been shown to be a major mechanism controlling these cell-fate choices. So far, the pleiotropy of *Delta* and *Jagged*/*Serrate* genes has hindered the evaluation of the roles of specific Notch ligands, as the phenotypes of knock-out mice are lethal before complete pancreas differentiation. Analyses of gene expression and experimental manipulations of zebrafish embryos allowed us to determine individual contributions of Notch ligands to pancreas development. We have found that temporally distinct phases of both endocrine and exocrine cell type specification are controlled by different *delta* and *jagged* genes. Specifically, *deltaA* knock-down embryos lack alpha cells, similarly to *mib* (Delta ubiquitin ligase) mutants and embryos treated with DAPT, a gamma secretase inhibitor able to block Notch signaling. Conversely, *jagged1b* morphants develop an excess of alpha-cells. Moreover, the pancreas of *jagged2* knock-down embryos has a decreased ratio of exocrine-to-endocrine compartments. Finally, overexpression of Notch1a-intracellular-domain in the whole pancreas primordium or specifically in beta-cells helped us to refine a model of pancreas differentiation in which cells exit the precursor state at defined stages to form the pancreatic cell lineages, and, by a feedback mediated by different Notch ligands, limit the number of other cells that can leave the precursor state.

Keywords: Zebrafish; Pancreas; Notch; Delta; Jagged; *mib*

Introduction

The vertebrate pancreas is constituted by an association of three different tissues: islets that control glucose homeostasis, exocrine acini that produce digestive enzymes, and duct cells that convey those enzymes to the gut lumen. As the lack of insulin causes diabetes, and pancreatic cancer is one of the most invasive and fatal carcinomas, the pancreas has become a subject of intense biomedical research in cell differentiation (Edlund, 2002). Pancreas differentiation relates to a major goal in developmental biology, which is to understand how cells with a similar developmental potential can take different fates. The main genetic mechanism used in metazoans for such cell-fate decisions is lateral specification. In this process, components of the Delta-Jagged/Notch signaling pathway are involved in the generation of different cellular subtypes from precursor populations (Artavanis-Tsakonas et al., 1999). Notch and its ligands, Delta and Jagged, are integral membrane proteins transmitting signals between neighbouring cells. In many cases, the interacting cells have a similar developmental potential. A cell that produces more ligand inhibits ligand production in its neighbors, and concomitantly the weaker inhibitory feedback from its neighbors enables the cell to increase its ligand production. The effect of this loop is to drive neighbouring cells into different fates (Haddon et al., 1998a; Lewis, 1998). The Notch signaling cascade has been shown to be important in pancreas development for both the maintenance of the pool of undifferentiated precursors and for

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exocrine differentiation (Apelqvist et al., 1999; Esni et al., 2004; Hald et al., 2003; Jensen et al., 2000). Notably, recent findings show that Notch has a normal inhibitory role in the regulation of acinar differentiation (Esni et al., 2004). These findings, together with the observation that multiple cell types of the pancreas form asynchronously (Argenton et al., 1999; Biemar et al., 2001; Field et al., 2003; Ohlsson et al., 1991; Slack, 1995), led to the hypothesis that Notch signaling regulates, in a temporally controlled fashion, several instances of pancreatic cell type specification (Murtaugh et al., 2003). Nonetheless, the contribution of each *Delta* or *Jagged* gene to cell differentiation in the pancreas has not been analyzed yet, as analysis of mutant mice is hampered by pleiotropy of phenotypes. Indeed, Dll1 or RBPJ-k knock-out cause death in null-mice between E9.5 and 12.5, before effects on beta-cells and acinar differentiation can be determined (Murtaugh et al., 2003). Here, we have investigated the role of Notch signaling in the pancreas using zebrafish, which is a suitable model to study endoderm development and differentiation (Bally-Cuif et al., 2000; Biemar et al., 2001; Crosnier et al., 2005; Dickmeis et al., 2001; Field et al., 2003; Tiso et al., 2002). Zebrafish pancreas development has been described in detail both morphologically and at the molecular level (Argenton et al., 1999; Biemar et al., 2001; Field et al., 2003; Zecchin et al., 2004). An early posterodorsal bud, containing only endocrine cells, develops progressively from the 12-somite stage (15 hpf, *h*ours *p*ost *f*ertilization) onward; beta-cells, expressing *preproinsulin*, are the first endocrine cells to appear at 12-somite stage, followed, at the 24-somite stage (21 hpf), by alpha-cells expressing *glucagon*. Later in development, at 32 hpf, a second, anteroventral bud develops and its appearance is characterized by the expression of *ptf1a*. The second bud originates the exocrine components as well as some endocrine cells (Field et al., 2003; Lin et al., 2004; Zecchin et al., 2004). Thus, the sequential appearance of multiple pancreatic cell types in zebrafish offers the opportunity to dissect the role of different Notch ligands in pancreatic differentiation. While mice have three *delta* genes, zebrafish have four: *deltaA, deltaB, deltaC* and *deltaD* (Haddon et al., 1998b). Moreover, zebrafish have three *jagged*-like genes: *jagged1a, jagged1b* and *jagged2* (Zecchin et al., 2005, see also Lorent et al., 2004) while mice have only two. As *jagged1a* and *deltaA* locate nearby on chromosome 1, and *jagged1b* and *deltaD* locate nearby on chromosome 13, the abundance of Notch ligands in zebrafish is likely to be the outcome of the genome duplication event that yielded two *jagged1* genes (*a* and *b*) and two *dll1* genes (*deltaA* and *deltaD*) (Zecchin et al., 2005). Hence, this provides the opportunity to study Notch ligand function in a model which is less affected by pleiotropy of phenotypes as the partial genetic redundancy of zebrafish genome has led to sub-functionalization (Force et al., 1999). In this work, we have dissected the specific contributions of Notch ligands to pancreas development and designed a model that depicts the role of Notch pathway in both maintaining the precursor state and controlling sequential segregation of different pancreatic cell types from precursor populations.

Material and methods

Fish maintenance and microinjections

Embryos were grown and staged at 28.5°C according to standard procedures (http://ZFIN.org). The *mib*^{ta52b} (Itoh et al., 2003) and *deltaA*^{dx2} (Appel et al., 1999) mutant strains were kindly provided by Ajay Chitnis and Bruce Appel, respectively. The *glucagon*:*GFP* and *nk2.2a*:*GFP* transgenic lines will be published elsewhere (S.P. and F.A., in preparation). The *ins:gal4* transgenic line (allele designation *m1080*) drives expression of a mini-*gal4* (nts 358–853 and 2382–3006 of Gal4 GenBank Z73604) from 1,2 kb of the zebrafish insulin promoter and transcription start site. *deltaD* (Takke and Campos-Ortega, 1999) and Xdelta-DN (Chitnis and Kintner, 1996) mRNAs were synthesized by linearization of the expression vectors and transcription with SP6 RNA polymerase (mMessage mMachine in vitro transcribed kit, Ambion, Austin, TX). Morpholino antisense oligos were obtained from Gene Tools.

The sequences of morpholinos used in our experiments were as follows:

deltaA^{ATG}MO, 5′-CTTCTCTTTTCGCCGACTGATTCAT-3′;
deltaA^{spl}MO, 5′-CATCAGCATTACTCACCGGCCACGT-3′;
deltaA^{mism}MO, 5′-CATgAGaATTACTgACCGGCaAgGT-3′;
jagged1b^{ATG}MO, 5′-ATACTGAACTCCGTCGCAGAATCAT-3′;
jagged1b^{spl}MO, 5′-AATCCTGCTACTCACTTTCACTGGC-3′;
jagged1b^{mism}MO, 5′-ATAgTGAAgTCCcTCGCAcAATgAT-3′;
jagged2^{ATG}MO, 5′-GATACAATTCCACATGCCGCCGATT-3′;
jagged2^{spl}MO, 5′-AGACGAATACTCACTGTTCTAGTGG-3′
jagged2^{mism}MO, 5′-GATAgAATTgCAgATGCCcCCcATT-3′.
(mismatched nucleotides are in small letters).

The stock solution was diluted to working concentrations of 0.5–2 mg/ml in Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM Ca $(NO_3)_2$, 5 mM HEPES, pH 7.6), before the injection into the yolk of one-cell stage embryos. In all microinjection experiments, mRNA encoding native GFP and phenol red were used to check the efficiency of the procedure. RT-PCR on total RNA extracted from 50 embryos was used to check the efficiency of splicing morpholinos. Notch signaling was inactivated in vivo by *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester (DAPT, Calbiochem) treatment (Geling et al., 2002). Briefly, DAPT is dissolved in DMSO to prepare a 10 mM stock solution. The stock solution is added to the embryos in order to reach a final concentration of 100 μM DAPT. Control embryos are treated with an identical amount of DMSO.

Heat shock experiments

For Notch gain-of-function analyses, embryos were obtained from matings between carriers of the *hsp70:Gal4* and *UAS:N*^{ICD} transgenes (Scheer and Campos-Ortega, 1999). *Notch1aICD* overexpression was induced by transferring embryos to 40°C medium for 30 min. To prevent high mortality due to sensitiveness of embryos at early stages, heat shocks at 12 hpf were performed in 39°C medium for 20 min. Following heat shock, embryos were incubated at 28.5°C until fixation. When the fixation was planned later than 12 h after the heat shock, one or more intermediate heat shocks were performed at intervals of 10–12 h in order to guarantee a continuous expression of Notch1aICD. After *in situ* hybridization and documentation, embryos were digested by proteinase K and genotyped by PCR using the following primers:

Gal4-F 5′-CGCTACTCTCCCAAAACCAAAAGG;
Gal4-R 5′-TCTCTTCCGATGATGATGTCGCAC;
UASNIC-F 5′-CATCGCGTCTCAGCCTCAC;
UASNIC-R 5′-CGGAATCGTTTATTGGTGTCG.

Whole-mount in situ hybridization and immunohistochemistry

Embryos were staged according to Kimmel et al. (1995). Whole-mount in situ hybridizations were performed according to Thisse et al. (1993). Whole-mount immunohistochemistry on *mib* embryos and wt siblings were performed with guinea pig anti-porcine insulin (DAKO) and the rabbit anti-porcine

glucagon (DAKO) antisera. The staining protocol has been previously described in detail (Argenton et al., 1999). Stained embryos were scanned and analyzed with a Leitz 510 confocal microscope and cell identification was aided by counterstain of nuclei with Hoechst 33342. Double staining whole mount in situ hybridization was carried out according to Hauptmann and Gester (Hauptmann and Gerster, 1994). The following digoxygenin- or fluorescein-labelled (Roche) antisense riboprobes were used: *insulin*, *somatostatin (PPSS2)*, *glucagon* and *pdx1(ipf1)* (Argenton et al., 1999); *ptf1a* (Zecchin et al., 2004); *mnr2a* (Wendik et al., 2004); *trypsin* (Biemar et al., 2001); *pax6.2 (pax6b)* (Nornes et al., 1998); *neuroD,* (Strahle and Blader, 1994); *ngn3* (Wang et al., 2001); *pept1(slc15a1)* (Verri et al., 2003); *deltaA* (Haddon et al., 1998b); *jagged1b* and *jagged2* (Zecchin et al., 2005). Enteroendocrine cells have been revealed in *glucagon*:*GFP*-transgenic embryos and *nkx2.2a*:*GFP* transgenic embryos (S.P. and F.A., manuscript in preparation) by using a *GFP* antisense probe. Control and morpholino- or mRNA-injected embryos have been hybridized in the same tube. The control embryos had the tip of the tail cut. Following in situ hybridization, embryos were post-fixed in 4% buffered p-formaldehyde and mounted in 85% glycerol/phosphate-buffered saline (PBS) for microscope observation. Embryos at early somitogenesis were dehydrated with methanol and cleared with a 2:1 mixture of benzylbenzoate:benzyl alcohol. Sections were obtained from embryos embedded in 4% agarose in PBS. The agarose blocks were sectioned in PBS using a Leica VT1000S vibratome. The slices were mounted in 50% glycerol/PBS for microscope observation. Observations were made with a Leica DMR compound/Nomarski microscope and images were acquired with a Leica DC500 digital camera. Images were processed using the Adobe Photoshop software.

Results

Endocrine and exocrine pancreatic differentiation is controlled by Notch signaling

Recent studies in mouse and zebrafish highlighted the involvement of Notch signaling in pancreatic differentiation (Apelqvist et al., 1999; Esni et al., 2004; Lorent et al., 2004; Murtaugh et al., 2003). As a first step to investigate the role of Notch signaling in the development of zebrafish pancreas we analyzed the differentiation of endocrine cells in *mib* mutants. *mib* mutants lack a Delta-ubiquitin ligase resulting in the failure to release Delta-mediated Notch signalling (Itoh et al., 2003). By in situ hybridization we found that *mib* mutants displayed an excess of beta- and delta-cells along with a reduced number or complete absence of alpha-cells (Figs. 1C, G, K). Anti-hormone immunostaining of 72 hpf *mib* mutants confirmed that increased number of beta- and delta-cells is accompanied by a decrease or lack of alpha-cell differentiation and glucagon production (Table 1). Indeed, the increased number of beta-cells in *mib* mutant embryos is observed as early as 24 hpf (not shown). Ligand binding to Notch triggers the cleavage of its intracellular domain (N^{icd}) by gamma-secretases (De Strooper et al., 1999). Then, N^{icd} translocates to the nucleus where it regulates the expression of Notch target genes. It has been already shown that treatment of wild type embryos with DAPT (a gamma-secretase inhibitor) phenocopies some aspects of the *mib* phenotype such as fused somites, increased neurogenesis and lack of pigmentation (Geling et al., 2002). We therefore tested whether DAPT treatment also affects pancreatic differentiation. As shown in Figs. 1D, H, L and C, G, K pancreatic

Table 1
Insulin, glucagon and somatostatin expressing cells in wild type and *mib* mutant embryos analyzed at 72 hpf

	Insulin-producing cells	Glucagon-producing cells	Somatostatin-producing cells
wt	45.9 (±5.2), $n=10$	51.0 (±5.6), $n=10$	50.4 (±5.3), $n=7$
mib	69 (±6.4), $n=4$	0.7 (±0.4), $n=3$	65.3 (±7.1), $n=3$

Quantitative confocal analysis of fluorescent embryos immunostained with anti-insulin, anti-glucagon and anti-somatostatin antibodies. Data are presented as average number of expressing cells (±standard error). The number of embryos analyzed for each cell type is indicated (n).

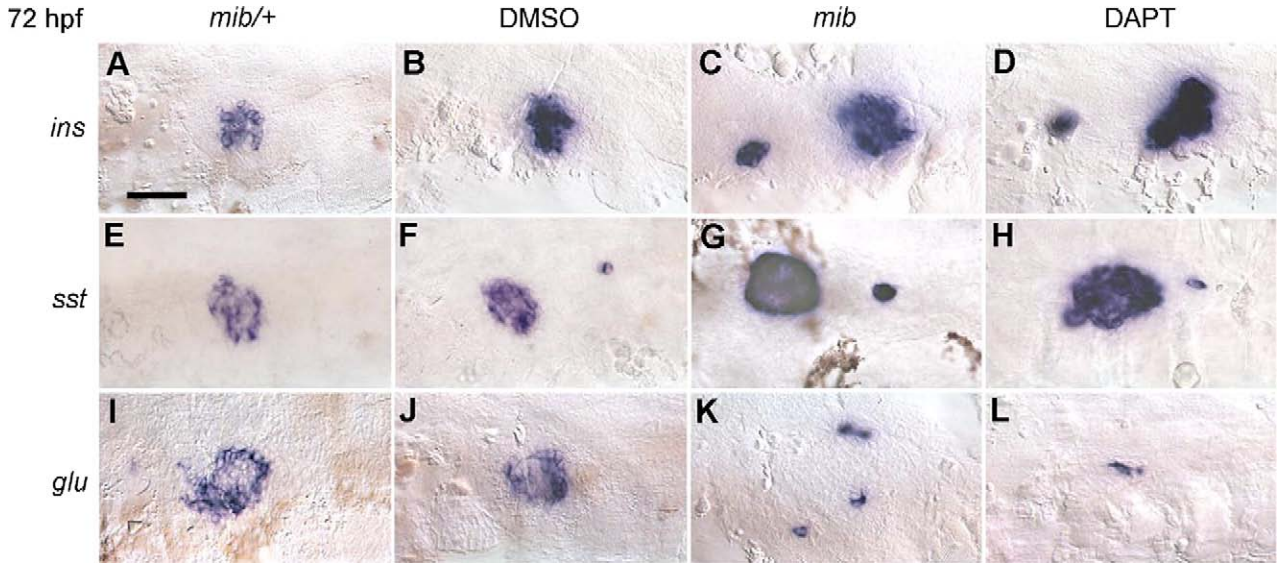


Fig. 1. Inhibition of Notch signalling increases differentiation of beta-cells. *In situ* hybridizations showing *insulin* (A–D) *somatostatin* (*PPSS2*) (E–H) and *glucagon* (I–L) expression in *mib* mutants and DAPT-treated embryos. The pancreatic area is depicted. *Insulin* and *somatostatin* expression, in blue, is increased in *mib* mutants and DAPT-treated embryos (C, D, G, H) compared with controls (A, B, E, F). Conversely, *glucagon* expression is reduced or absent (compare K, L with I, J). Embryos in panels A, E, I and B, F, J should be compared with embryos in panels C, G, K and D, H, L, respectively. Embryos have been hybridized at 72 hpf and are presented in a ventral view with anterior to the left. Scale bar in panel A is 50 μm.

Table 2
Effect of DAPT treatment at different stages on the differentiation of alpha- and beta-cells

Embryos number	Glucagon expressing cells (fold) compared to the average number in controls			Time of DAPT addition	Insulin expressing cells (fold) compared to the average number in controls			Embryos number
	1×	0.5–0.1×	<0.1×		1×	1.5×	1.5–2×	
14	_	21%	79%	2–4 cell	7%	_	93%	14
34	_	15%	85%	5 hpf	21%	36%	43%	33
46	11%	30%	59%	8.5 hpf	39%	16%	45%	44
42	45%	26%	29%	13 hpf	95%	5%	_	43

Embryos have been treated continuously with 100 μM DAPT from 2 to 4 cells, 5, 8.5 or 13 hpf stages and analyzed, by in situ hybridization, at 33 hpf. The number of *insulin* and *glucagon* expressing cells is compared to that of control embryos of the same clutch, treated with DMSO only. The vast majority of the embryos treated with DAPT from 2 to 4 cell stage or 5 hpf show increase in the number of *insulin* expressing cells. The proportion is decreased when embryos are treated with DAPT from 8.5 hpf and minimal when treated from 13 hpf. Glucagon producing cells decrease in number as insulin producing cells increase.

endocrine cell differentiation is indistinguishable in *mib* embryos and embryos treated with DAPT from the 2-cell stage. In both cases the block of Notch signaling is accompanied by an increased number of beta- and delta-cells, but lack of alpha-cell differentiation and *glucagon* expression. In order to determine when Notch signaling is acting in pancreas development, we started DAPT treatment at different time points and counted the number of insulin- and glucagon-producing cells at 30 hpf. Our data, summarized in Table 2, indicate that endocrine differentiation progresses between 5 and 13 hpf and starts to be controlled by Notch signaling between 5 hpf and 8.5 hpf.

We then examined exocrine pancreas differentiation in *mib* mutants and DAPT-treated embryos by analyzing the expression of genes such as *mnr2a*, *ptf1a* and *trypsin* (Biemar et al., 2001; Wendik et al., 2004; Zecchin et al., 2004). We observed that at 40 and 72 hpf, the number of exocrine cells in the pancreas is reduced in *mib* mutants (Figs. 2C, G, K) as well as in embryos treated with DAPT from 2 hpf (Figs. 2D, H, L). As treatment with DAPT at blastula stages does not change mesendodermal segregation and the total amount of endoderm (Kikuchi et al., 2004), our results are consistent with the idea that impairment of Notch signaling in *mib* mutants favors the early pancreatic fates at the expense of the late ones. In addition, the reduction of the exocrine tissue was still observed even if DAPT treatment was started at the 5-somite stage, a stage at which endoderm segregation from mesendodermal precursors is likely to be completed (data not shown). However, as the time lag of DAPT penetration and activity is unknown, the different effects of DAPT on endocrine and exocrine differentiation in the time scale can be taken only as a simple temporal sequence.

We further investigated, after modulation of Notch signaling, the expression of a panel of nuclear factors transcribed in pancreatic precursors. *pdx1* is a gene expressed in the duodenum and its activity is required for the development of all cellular components of the pancreas (Ohlsson et al., 1991; Yee et al., 2001). It can be noted that the endocrine islet in *mib* mutants is somewhat enlarged at 28 hpf (Fig. 3B) and clearly increased in size at 48 hpf (Fig. 3D). Moreover, at the latter stage a larger number of cells express *neuroD* and *pax6.2* which are markers for endocrine precursors (Figs. 3E–H). Notably, in *mib* mutants analyzed at 70 or 96 hpf, the expression of *ngn3, pax6.2* and *neuroD* was markedly expanded in the gut (Figs. 3I–N). This result is reminiscent of the effect of the *Hes1*-null mutation in mouse that, as revealed by expression of *NeuroD, Pax6* and *Ngn3*, causes an accelerated differentiation of the endocrine cells of the gut (Jensen et al., 2000). Similarly, we found that a *glucagon:GFP*

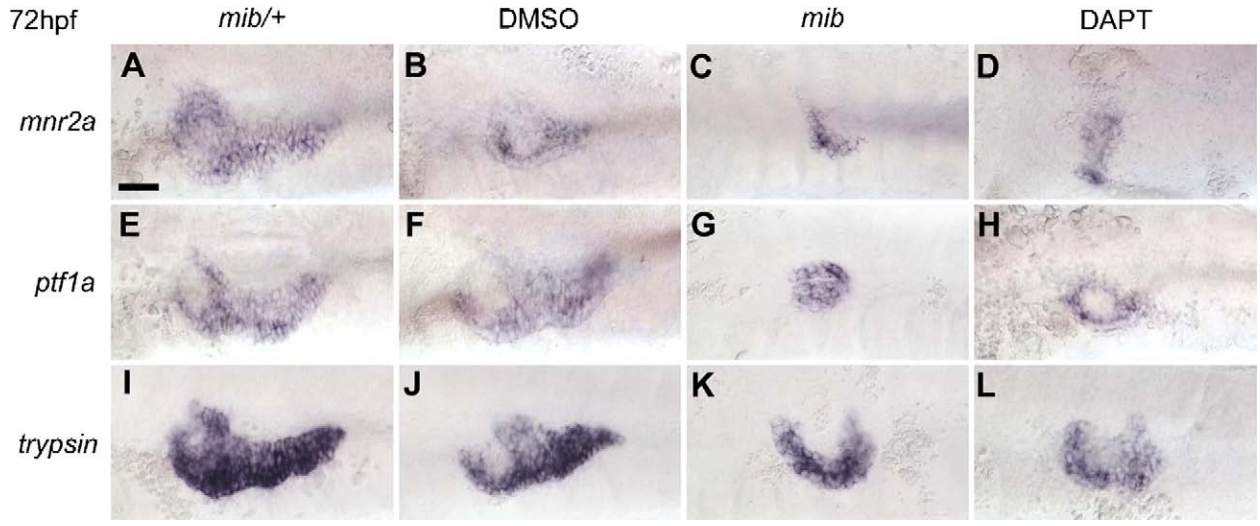


Fig. 2. Block of Notch signalling decreases the number of exocrine cells in the pancreas. *In situ* hybridizations showing reduction of *mnr2a* (A–D), *ptf1a* (E–H) and *trypsin* (I–L) expression in *mib* mutants (C, G, K) and DAPT-treated embryos (D, H, L). The pancreatic area is depicted. Embryos in panels A, E, I and B, F, J should be compared with embryos in panels C, G, K and D, H, L, respectively. Embryos have been hybridized at 72 hpf and are presented in ventral view with anterior to the left. Scale bar in panel A is 50 μm.

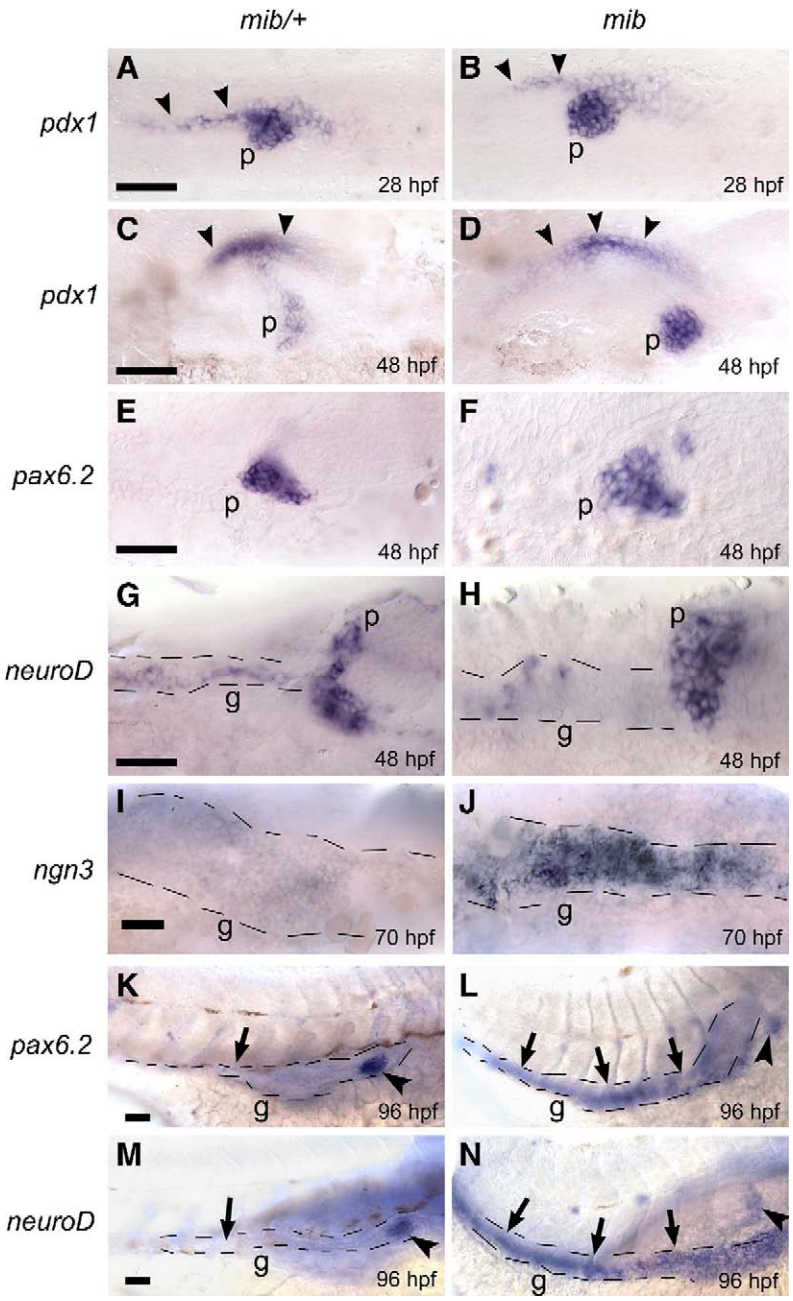


Fig. 3. Endoderm development in *mib* mutants. In *situ* hybridizations showing expression of *pdx1*, *pax6.2, neuroD* and *ngn3* in the endoderm of *mib* mutant embryos and controls. Panels A–D: *pdx1* is expressed at normal levels in the gut (arrowheads) of *mib* embryos and their wild type siblings, whereas its expression is stronger in the endocrine pancreas (p) of *mib* mutants (B, D) when compared with controls (A, C). Panels E–H: the expression of *neuroD* and *pax6.2,* both active in endocrine precursors, is stronger in the endocrine pancreas (p) of *mib* mutants (F, H) when compared with controls (E, G). The gut (g) is outlined. Panels I–J: *ngn3* is expressed ectopically in the gut (g, also outlined) of *mib* mutants. Panels K–N: *neuroD* and *pax6.2* are expressed ectopically in the gut (g, indicated by arrows and outlined) of *mib* mutants (L, N). The endocrine pancreas (arrowhead) is indicated. Embryos are presented in ventral (A–J) or lateral (K–N) views with anterior to the left (A–J) or to the right (K–N). Scale bar is 50 μm.

transgenic line, in which the *GFP* is driven by the zebrafish *glucagon* promoter, treated with DAPT at 30 hpf and analyzed at 75 hpf, develops an excess of cells expressing GFP in the gut; this can be assessed either by fluorescence microscopy (not shown) or by *in situ* hybridization with a *GFP* probe (Figs. 4A, B). Accordingly, a *nxk2.2a:GFP* transgenic line, in which the *GFP* is under the control of the zebrafish *nkx2.2a* promoter, shows an increased number of enteroendocrine precursors in the gut (Figs. 4C, D) when treated with DAPT at 30 hpf. Moreover, *mib* mutants, analyzed by *in situ* hybridization at 96 hpf, display an excess of *glucagon*-expressing cells in the intestine (Figs. 4E, F). These results demonstrate that in zebrafish, like in mammals, an excess of enteroendocrine cells differentiates in the gut when Notch signaling is inhibited. Conversely, the absence of *pept1* gene, which encodes a peptide transporter expressed in the intestinal epithelium (Verri et al., 2003), reveals that the differentiation of enterocytes is impaired in *mib* mutant embryos (Figs. 4I–J).

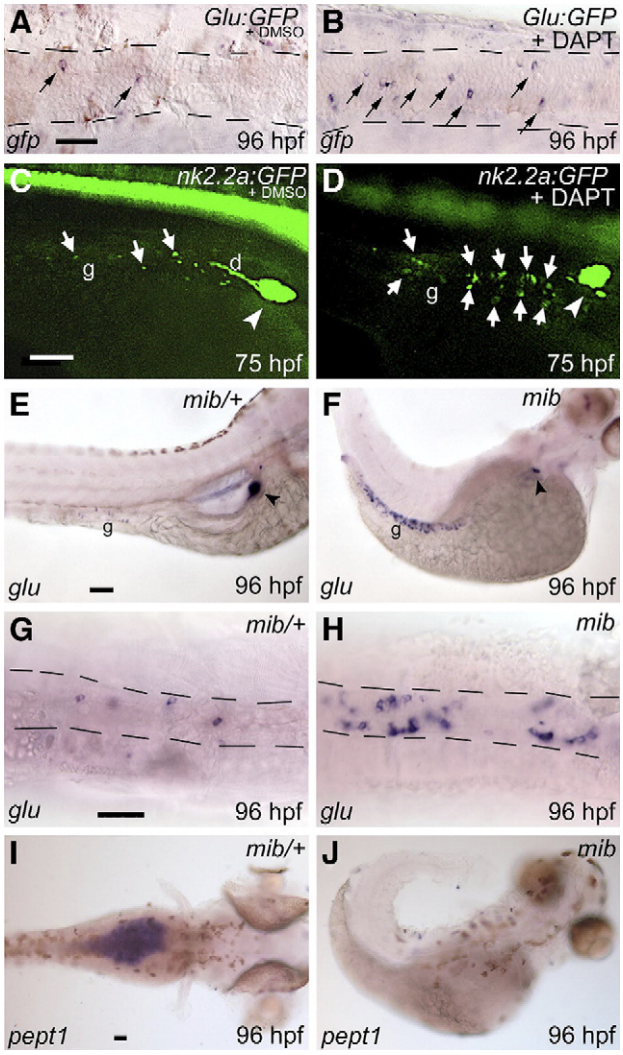


Fig. 4. Increase of enteroendocrine cell differentiation in *mib* mutants and DAPT-treated embryos. Panels A, B: *in situ* hybridization with a *GFP* antisense probe showing increased activity of the *glucagon* promoter in the gut (black arrows) of a *glucagon:GFP* (*glu*:GFP) transgenic zebrafish line treated with DAPT at 30 hpf (B). Panels C, D: *nk2.2a:GFP* transgenic embryo showing an increase of enteroendocrine cells (white arrows) when treated with DAPT at 30 hpf (D). The pancreatic duct (d) and the endocrine islet (arrowhead) are indicated. Panels E–H: *glucagon* expression in the gut (g) and pancreas (black arrowhead) of wt (E, G) and *mib* mutants (F, H) analyzed at 96 hpf. In panels G–H the gut is outlined. Panels I, J: expression of the peptide transporter *pept1* in wt (I) and *mib* mutant embryos (J). Embryos are in lateral (C–F), ventral (A, B) or dorsal (G–J) views with anterior to the right. Scale bar is 50 μm.

Expression of specific Notch ligands in pancreatic development

The involvement of Notch-signaling in pancreatic development is well documented, but, so far, the function of specific Notch ligands has been elusive. To address this issue we have analyzed the pancreatic expression of all known Notch ligands throughout embryonic and early larval stages, when all the known components of the pancreas differentiate (Biemar et al., 2001). The pancreatic expression patterns of the four *delta* (A–D) (Haddon et al., 1998b) and the three *jagged* (1a, 1b and 2) (Haddon et al., 1998a; Zecchin et al., 2005) genes have been analyzed between 5-somite and 48 hpf, at crucial stages of pancreas differentiation. We have found that only *deltaA*, *jagged1b* and *jagged2* are expressed in the pancreatic region of the endoderm. *deltaA,* which is the earliest Notch ligand expressed in the pancreatic primordium, is first detectable at the 7-somite stage in few endodermal cells at the level of the second somite. Its expression pattern coincides temporally and spatially with that of *neuroD*, one of the earliest proneural markers detectable in the pancreatic area (Figs. 5A, B). From the 7-somite stage onwards, the expression levels and number of cells expressing *deltaA* decrease until 30 hpf. Notably, in some cells of the pancreas, *deltaA* has an expression domain overlapping with that of *insulin* and *pdx1* (Figs. 5C–F). The time span of *deltaA* expression encompasses the stages of differentiation of alpha- and beta-cells. *jagged1b,* which is the second Notch ligand expressed in the pancreatic primordium, was first detected anteriorly and around beta-cells at 28 hpf (Figs. 5G, H and Zecchin et al., 2005). Remarkably, alpha-cells differentiate during the time-span of *jagged1b* expression but, as already reported, its expression could not be detected in differentiated alpha-cells (Zecchin et al., 2005). We have not been able to detect *jagged1b* expression by *in situ* hybridization in the pancreas at earlier or later stages up to 4 dpf of development. Finally, we detected the expression of *jagged2* in the pancreatic primordium at 36 hpf, in the *ptf1a*-expressing domain that will originate the anteroventral bud (Figs. 5I, J and Zecchin et al., 2005).

Functional role of specific Notch ligands in pancreas development

The specific developmental stages and pancreatic sites of expression of *deltaA*, *jagged1b* and *jagged2* suggest their involvement in pancreatic differentiation. In order to dissect their specific function, we have knocked-down the expression of each ligand independently.

The early expression of *deltaA* in the pancreatic primordium, which is similar to *neuroD* expression, points toward a specific role of this Notch ligand in the differentiation of alpha- and beta-cells. When tested for *insulin* expression, dlA^{dx2} mutants revealed only a small change in the number of *insulin*-expressing cells. As the *dx2* allele of *deltaA* is a dominant, incompletely penetrant missense mutation (Appel et al., 1999), we also tested the effect of antisense morpholinos to *deltaA* on *glucagon* and *insulin* expression. As shown in Figs. 6A–D, in embryos injected with an antisense morpholino targeting *deltaA* translation ($deltaA^{ATG}$MO) the number of alpha-cells is dramatically decreased while the number of beta-cells is significantly increased. Moreover, injection of a morpholino directed against the splice-site of *deltaA* also caused a strong decrease of *glucagon*-expressing cells (data not shown). Hence, it appears that DeltaA is required for the specification of the proper number of alpha-cells. The shift towards beta-cells may indicate that DeltaA acts on a common precursor pool for both cell types. Expression of *ptf1a*, a gene expressed in the anteroventral bud of the pancreas, is normal in *deltaA* morphants analyzed at 48 hpf. However, in *deltaA* morphants analyzed at 72 hpf, *trypsin* expressing cells, although present, are decreased in number (data not shown).

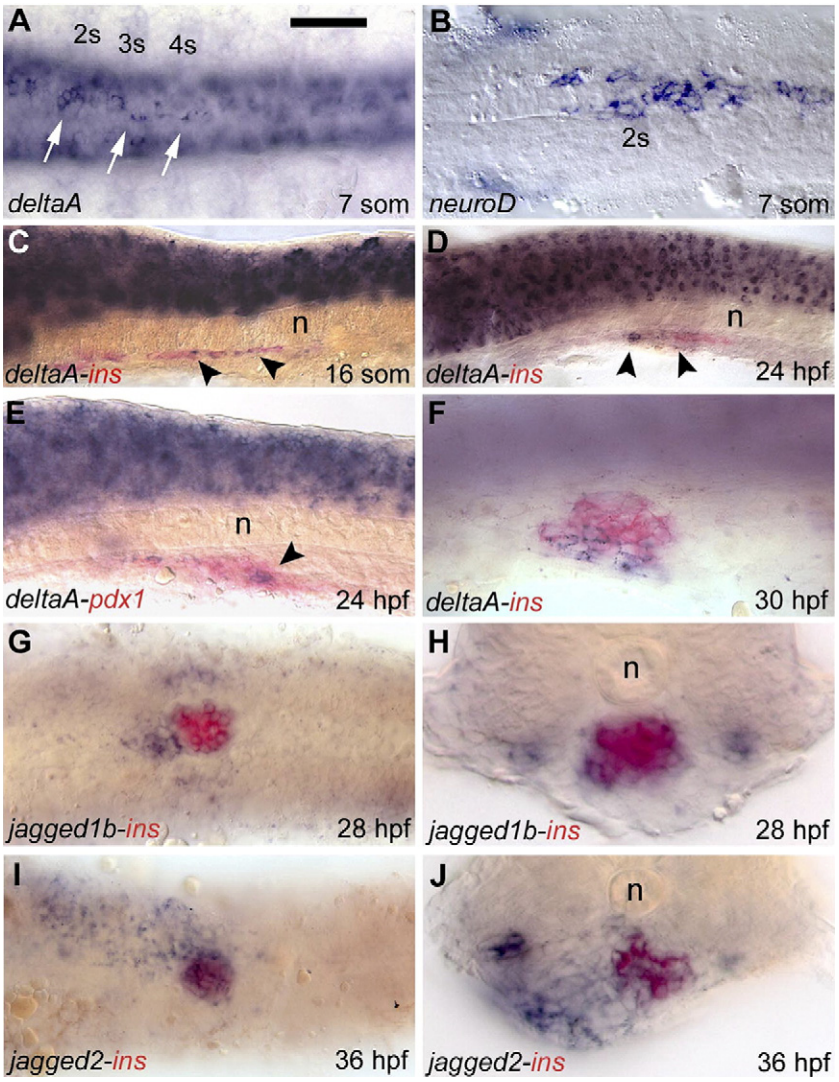


Fig. 5. Expression of Notch ligands in the pancreatic primordium. Panel A: *deltaA* expression in the endoderm of an embryo at the 7-somite stage. Panel B: expression of *neuroD* in the endoderm of an embryo at the 7-somite stage. Panels C, D, F: expression of *insulin* (red) and *deltaA* (blue) in the endoderm of zebrafish embryos. Panel E: expression of *pdx1* (red) and *deltaA* (blue) in the endoderm of zebrafish embryos at 24 hpf. Panels G, H: expression at 28 hpf of *jagged1b* (blue) in cells located around the *insulin* (red) producing cells. Panels I, J: expression of *insulin* (red) and *jagged2* (blue) in the endoderm of zebrafish embryos at 36 hpf. The pancreatic primordium is indicated with a black arrowhead. "n" indicates the notochord; "2s" indicates the position of the second somite. Embryos are in ventral (A, B, G, I), lateral (C–F) views with anterior to the left or transversal vibratome sections (H, J) Scale bar in panel A is 50 μm.

The second Notch ligand expressed in the pancreatic area is *jagged1b*. By injecting a morpholino targeting ATG, we have knocked-down *jagged1b* expression and analyzed *insulin*, *glucagon* and *trypsin* expression in morphant embryos. The results show that while the number of *insulin*-expressing cells appears unaffected (not shown), the number of *glucagon*-expressing cells is increased (Figs. 6E, F) Raw data show that increase in the number of alpha cells becomes apparent from 48 hpf onwards (Table 3). Moreover, in *jagged1b* morphants, similarly to *mib* mutants, the number of *glucagon*-expressing cells of the gut is strongly increased (Figs. 6G–H) while the number of *trypsin*-expressing cells decreases (Figs. 6I–J).

Jagged2 expression was knocked-down with an antisense morpholino targeting the ATG. As shown in Figs. 6K–N and Fig. S1, staining of *jagged2*-morphants revealed that the total number of *insulin*- and *glucagon*-expressing cells was increased (Table 4) while the amount of exocrine tissue was reduced. This effect on the endocrine and the exocrine lineages suggests that the differentiation of cells arising from the second pancreatic bud is under the control of *jagged2* activity. Due to the fact that antibodies against zebrafish jagged1b and jagged2 proteins were not available, we have repeated our experiments of inactivation with morpholinos directed against splicing of both genes and whose knock-down activity could be easily checked by RT-PCR. Results are reported in Supplementary Material (Figs. S2, S3 and Table S1) and confirm the results obtained with morpholinos directed against ATG.

Validation of a model of Notch-mediated pancreatic differentiation in zebrafish

The results of the above-described experiments suggested that the segregation of pancreatic cell types from precursor

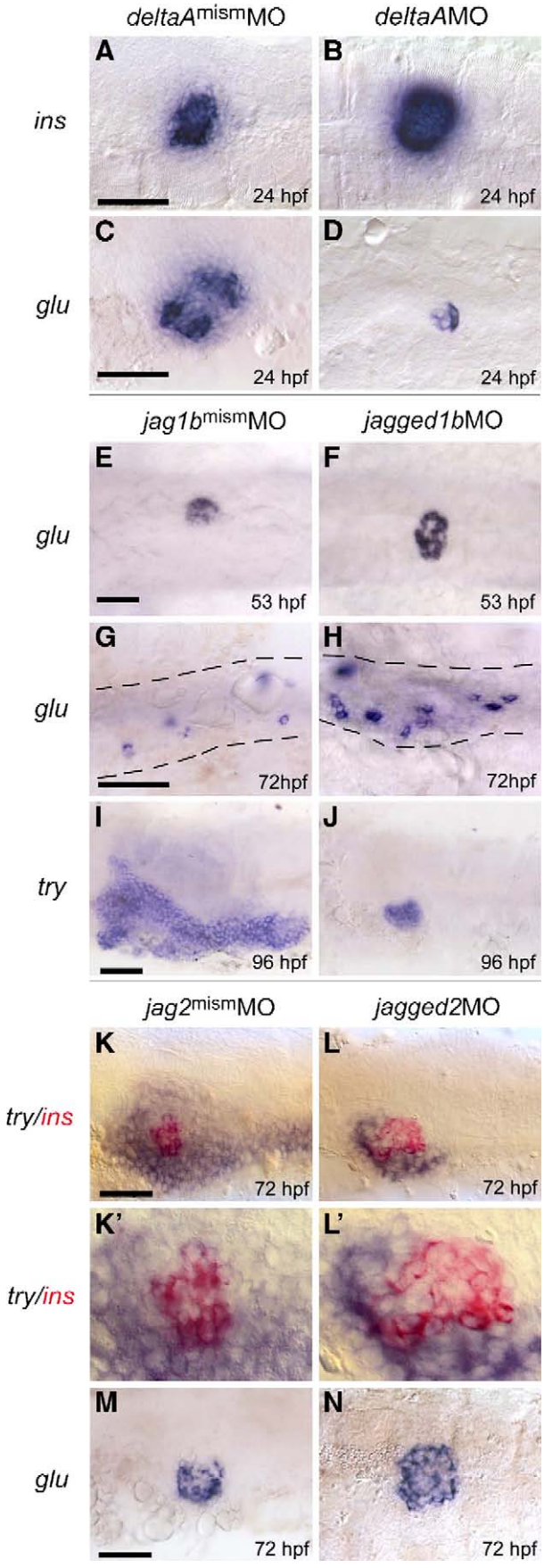


Table 3
Effect of *jagged1b* morpholino on the total amount of alpha cells of the pancreas

Embryos stage (hpf)	Glucagon expressing cells (fold) in *jagged1b* morphants compared to the average number in controls			Embryos number
	1×	1.5×	1.5–2×	
33	39	_	_	39
48	10 (25%)	30 (75%)	_	40
53	14 (36%)	25 (64%)	_	39
72	16 (47%)	_	18 (53%)	34

Fertilized zebrafish eggs have been injected with 10 ng of *jagged1b*-morpholino, fixed at the stage reported on the left column and hybridized with an antisense probe for *glucagon*. Data are expressed as increase in glucagon expressing cells compared to average levels, at the same timepoint, of control embryos injected with *jagged1b*-mismatched morpholino.

pools is sequentially assisted by three Notch-ligands expressed in the pancreatic primordium. A possible model to explain our findings is that a common pool of precursors may provide, discontinuously, groups of cells that exit the undifferentiated state. In particular, beta- and delta-cells differentiate between 15 and 24 hpf and alpha-cells between 21 and 30 hpf, from the posterodorsal bud, while the anteroventral bud (containing mainly exocrine precursors) differentiates from 33 hpf onwards. Trypsin expression is rarely observed before 40 hpf and true cytologic differentiation probably occurs even later (Esni et al., 2004; Yee et al., 2005). Specific *delta* or *jagged* genes, expressed by differentiating cells, could be essential to provide the pool of precursors with a negative feedback signal that blocks the differentiation of more cells. Thus, the intracellular release of Notch should inhibit differentiation and keep precursors in an undifferentiated state. To test this model we have taken advantage of transgenic embryos containing a heat shock-inducible *hsp70:Gal4* transgene, in combination with a Gal4-responsive *UAS:Notch1a-intracellular-domain* (N^{ICD}) allele (Scheer and Campos-Ortega, 1999). We first tested if N^{ICD} could block endocrine cell differentiation and increase the number of progenitors. It can be observed that in embryos heat-shocked at 12 hpf and analyzed at 24 hpf, alpha-, beta- and delta-cells are lacking (Figs. 7D′, G′ and J′), while the pancreatic progenitors, revealed by *pdx1*, are still present (Fig. 7A′). When N^{ICD} expression is induced at 20 hpf (after the onset of *deltaA* expression and before that of *jagged1b*), the number of beta- and delta-cells is reduced while alpha-cells are missing (Figs. 7E′, H′ and K′). This is likely to be

Fig. 6. Effects of morpholino-mediated knock down of Notch ligands on the differentiation of pancreatic cell populations. Panels A–D: *insulin* and *glucagon* expression at 24 hpf in control and *deltaA*-morphant embryos. *glucagon*-expressing cells are decreased in number in *deltaA* morphants (D). Panels E–H: *glucagon* expression in the pancreas (E, F) and gut (G, H) is increased in *jagged1b*-morphant embryos (F, H) compared with controls (E, G). Panels I–J: *trypsin* expression is decreased in *jagged1b* morphant embryos. Panels K–N: expression of endocrine and exocrine markers in *jagged2*-morphant embryos. *insulin* (red) expression is increased and *trypsin* (blue) expression decreased in *jagged2*-morphants (panels L and L′ blow up) compared with controls (panels K and K′ blow up). *glucagon* expression profile in *jagged2*-morphants is enlarged (N) reflecting the increased size of the beta-cell cluster. Embryos are in ventral view with anterior to the left. Stages are indicated at the bottom right of each frame. The scale bar is 50 μm.

Table 4
Effect of *jagged2* morpholino on the total amount of beta cells of the pancreas

Embryos stage (hpf)	Insulin expressing cells (fold) in *jagged2* morphants compared to the average number in controls			Embryos number
	1	1.5	1.5–2	
48	9 (17%)	19 (36%)	25 (47%)	53
72	–	–	20 (100%)	20

Fertilized zebrafish eggs have been injected with 10 ng of *jagged2*-morpholino, fixed at the stage reported on the left column and hybridized with an antisense probe for insulin. Data are expressed as increase in insulin expressing cells compared to average levels of control embryos injected with *jagged2*-mismatched morpholino and fixed at the same timepoint.

the result of the initiation of the heat shock after specification of early forming beta- and delta-cells. Again, following heat shock at 20 hpf, the *pdx1* expression revealed an increased number of progenitors (Fig. 7B′) while exocrine precursors fail to differentiate (Fig. S4). The block of endocrine differentiation by N^{icd} expression is also evidenced by the fact that heat shock at either 12 or 20 hpf blocks the expression of both *pax6.2* and *neuroD*, two markers of endocrine differentiation (Fig. S5). Moreover, when N^{ICD} expression is induced at 30 hpf, which is just before the start of *jagged2* expression, the three hormone-producing cells are present but *trypsin* expression is lacking and the levels of *ptf1a* are strongly reduced (Figs. 7M–P′). Finally, when Gal4 is expressed under the control of the *insulin* promoter, so that N^{ICD} is expressed only in insulin-producing cells, beta-cells are missing but alpha- and delta-cells are unaffected (Fig. 8B–D′). This demonstrates that the function of Notch signaling is not to decide between alternative cell fates but, rather, to inhibit differentiation.

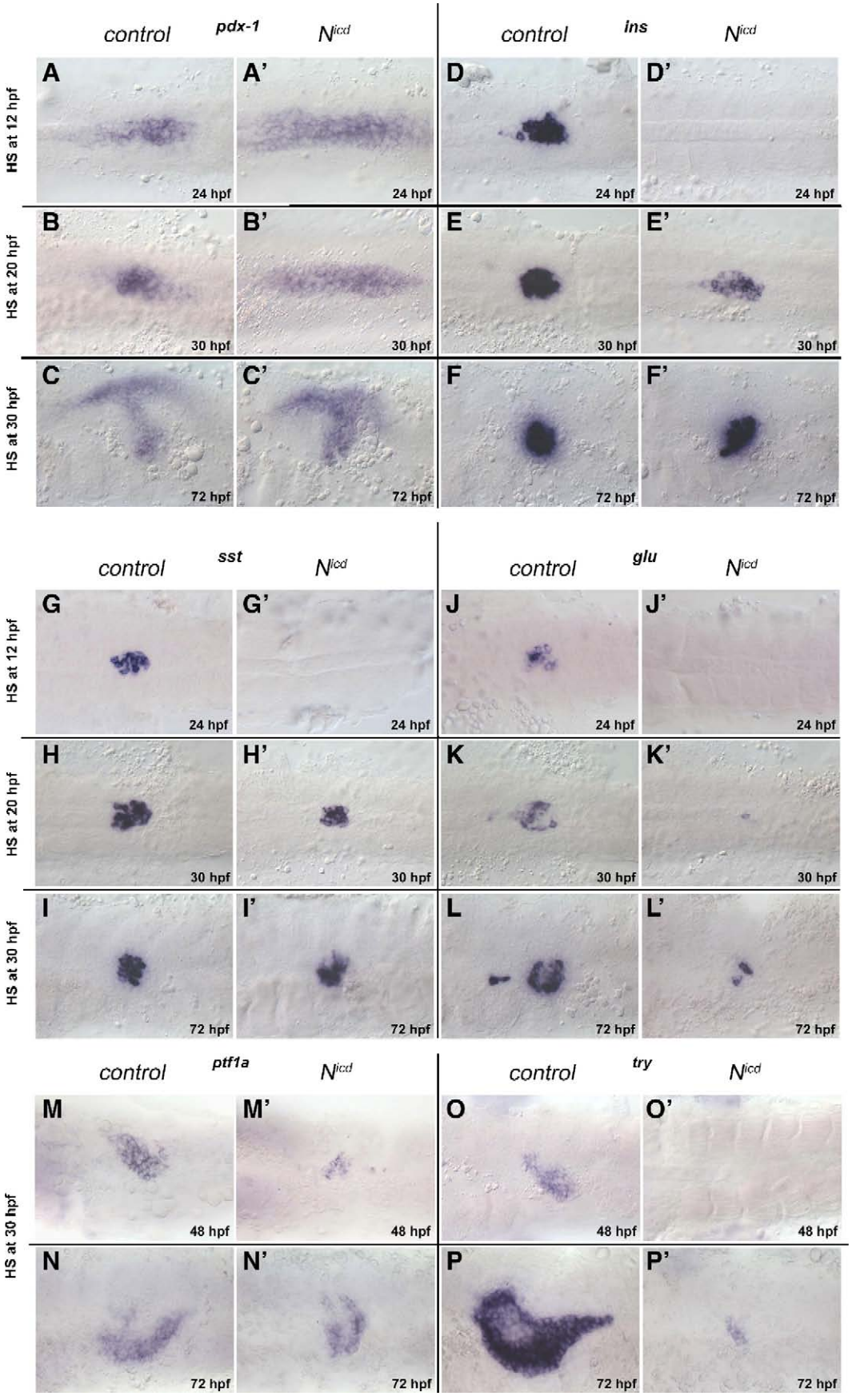
Discussion

Using a combined epigenetic and genetic approach we have analyzed the role of Notch signaling and its ligands during pancreatic differentiation in zebrafish. Our results are consistent with a model of pancreas differentiation in which a ligand-specific Notch-mediated feedback on precursors limits the number of committed cells (Fig. 9). In the first series of experiments, we have found that *mib* mutants, in which Notch signalling is impaired (Itoh et al., 2003), lack alpha-cells and have a strongly increased number of beta-cells. These results are confirmed with different endocrine markers and by persistence of *pdx1* expression in the *mib* islet. We obtained similar results in embryos treated with the gamma-secretase inhibitor DAPT. In the mouse, in which contrary to zebrafish the alpha-cells appear first, the lack of Notch signalling accelerates differentiation of alpha-cells and causes a depletion of pancreatic precursors with decreased number of beta-cells (Jensen et al., 2000). Thus, it seems that in zebrafish, like in mouse, the inhibition of Notch signalling causes a depletion of the pancreatic precursors due to increased differentiation of the earliest appearing endocrine cell type. In zebrafish, the two main pancreatic components derive from different endodermal buds. The first islet forms between 12 to 24 hpf from a posterodorsal bud. This islet is engulfed, from 36 hpf, by cells originating from an anteroventral bud, forming the exocrine tissue and additional endocrine cells (Field et al., 2003; Gnugge et al., 2004; Wendik et al., 2004; Zecchin et al., 2004). Thus, the early effect of DAPT on both the endocrine and exocrine pancreas compartments opens the possibility for a common origin of the pools of progenitors originating the endocrine and exocrine tissues i.e., Notch signaling may contribute to lineage decisions on a common progenitor/precursor pool before cells are allocated into the anteroventral and posterodorsal pancreatic aspects.

The gut phenotype of *mib* or DAPT-treated embryos displays close similarities with that of *Hes1* knock-out mice (Jensen et al., 2000): in both cases, the number of enteroendocrine cells is increased at the expense of enterocytes. These results confirm recent findings showing that Delta/Notch controls the commitment to a secretory fate during development of the zebrafish intestine (Crosnier et al., 2005). Using gut cell-type specific monoclonal antibodies, Crosnier and co-workers demonstrated that *mib* and *aei/deltaD* mutants have an excess of secretory cells at the expenses of enterocytes. Their findings point to DeltaD as one of the Notch ligands responsible for this Notch-dependent activity in the gut. It is remarkable that the zebrafish paralogues *deltaA* and *deltaD* are orthologues to a single Dll1 gene in the mouse. Thus, it is tempting to speculate that the two domains of endodermal activity for deltaA and deltaD (pancreas and gut, respectively) could be the outcome of gene duplication and sub-functionalization events. Notably, a similar sub-functionalization has already been described in zebrafish for *deltaA* and *deltaD* in the nervous system (Haddon et al., 1998b) and for a number of other pairs of paralogues as well (Force et al., 1999).

The temporal sequence of differentiation of the endocrine and exocrine components of the zebrafish pancreas made it possible to dissect the roles of Notch and its ligands at specific time-points. We have demonstrated that three different Notch ligands are necessary for a correct differentiation of the endocrine and exocrine cell types of the pancreas. *deltaA* is

Fig. 7. Expression of *pdx1*, *insulin*, *somatostatin (PPSS2)*, *glucagon*, *ptf1a* and *trypsin* in control (*hsp70:Gal4* neg; *UAS:*N^{ICD}pos or *hsp70:Gal4*pos; *UAS:*N^{ICD}neg) and N^{ICD}-expressing (*hsp70:Gal4*pos; *UAS:*N^{ICD}pos) embryos heat shocked at different time points. Markers analyzed are indicated at the top of each subset of panels, while the time point of the first heat shock (HS) is indicated on the left. For each gene expression test, mono-genic control embryos (*hsp70:Gal4* neg or *UAS:*N^{ICD}neg) are on the left and bi-genic embryos (*hsp70:Gal4pos* and *UAS:*N^{ICD}*pos*) are on the right. **pdx1** (A–C); precursors, expressing *pdx1*, are increased in number when bi-genic embryos are heat shocked at 20 hpf or before (A′, B′, C′) indicating that Notch activation leads to proliferation of precursors. **ins** and **sst** (D–I); differentiation of *insulin*- and *somatostatin*-producing cells is completely blocked only when bi-genic embryos are heat shocked at 12 hpf (D′ and G′). **glu** (J–L); differentiation of glucagon-producing cells is completely blocked when bi-genic embryos are heat shocked at 20 hpf or before (J′ and K′) and severely impaired in embryos heat shocked at 30 hpf (L′). **ptf1a** and **try** (M–P); bigenic embryos heat shocked at 30 hpf fail to differentiate the exocrine pancreas (M′, N′, O′, P′). The stage at which the embryos were analyzed is indicated within each frame. Individual embryos were genotyped by PCR for presence or absence of each transgene. Embryos are in ventral views with anterior to the left.


control
pdx-1
Nicd
control
ins
Nicd
HS at 12 hpf
HS at 20 hpf
HS at 30 hpf
A
A'
D
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E
E'
C
C'
F
F'
24 hpf
30 hpf
72 hpf
control
sst
Nicd
control
glu
Nicd
G
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I
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control
ptf1a
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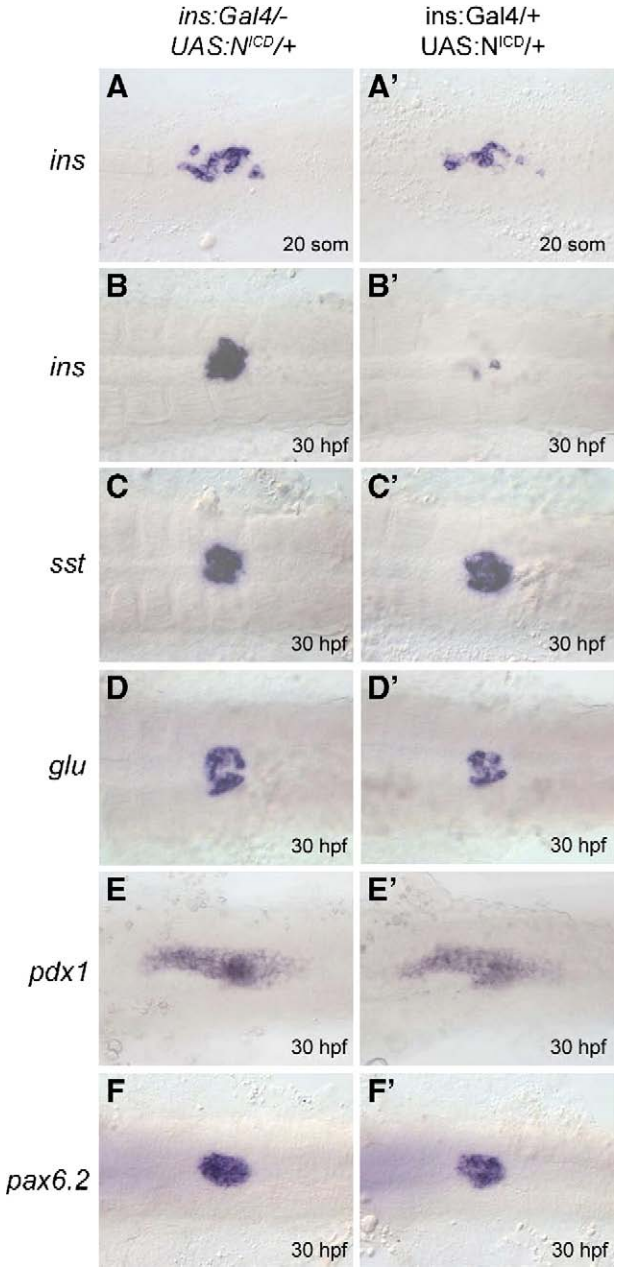

Fig. 8. Expression of *insulin*, *somatostatin (PPSS2)*, *glucagon, pax6.2* and *pdx1* in *ins*:Gal4;UAS:N^{ICD} bi-genic embryos. Mono-genic control embryos (*ins:Gal4* neg and *UAS:N^{ICD}* pos) are on the left and bi-genic embryos (*ins:Gal4* pos and *UAS:N^{ICD}* pos) are on the right. Expression of N^{ICD} under the control of the *insulin* promoter leads to the lack of *insulin*-producing cells but *somatostatin* and *glucagon* producing cells are present similarly to control. Genes whose expression is analyzed are indicated on the left while the genotypes are indicated on the top. Embryos are in ventral views with anterior to the left. Individual embryos were genotyped by PCR for presence or absence of each transgene. Scale bar is 50 μm.

expressed in the presumptive pancreatic endoderm at very early stages of differentiation, in regions where other specific genes (such as *neuroD*) are also expressed well before the onset of detectable hormone expression. *deltaA*-morphants have an increased beta-/alpha-cell ratio and their pancreatic phenotype is very similar to that of *mib* mutants and DAPT-treated embryos. This is consistent with a role of DeltaA in suppressing beta-cell differentiation in early endocrine precursors and thereby maintaining a suitable precursor pool available for the later differentiation of endocrine and exocrine lineages. The second Notch ligand expressed in the pancreatic region is *jagged1b*, which is expressed around beta-cells and anterior to the main islet (Zecchin et al., 2004). Its inactivation causes a marked increase of alpha-cells while the number of beta-cells is unaltered. In addition, the strong increase of endocrine cells in the gut of *jagged1b* morphants is a clue that Jagged1b might be the previously unknown Notch ligand that cooperates with DeltaD in intestinal differentiation (Crosnier et al., 2005). Interestingly, also *Hes1* knock-out mice have ectopic glucagon expression (Jensen et al., 2000) associated with the failure to form the biliary duct (Sumazaki et al., 2004). Thus, in *jagged1b*-morphants, the increased number of alpha-cells in the pancreas could also be explained with recruitment of cells of the biliary epithelium to an endocrine fate.

The third Notch ligand, *jagged2*, seems to be necessary to allow exocrine differentiation and its expression at 36 hpf in the *ptf1a*-positive gut epithelium is indeed consistent with this hypothesis. The bias towards endocrine fates at the expense of exocrine ones in *jagged2* morphants is most possibly due to a lack of feedback from endocrine cells just formed. Indeed, while it is still unclear to what extent the anteroventral bud contributes to the endocrine compartment at larval stages, data from Field et al. (2003) clearly shows that in wild type embryos analyzed at 76 hpf, a small secondary islet is formed from the anteroventral bud. Moreover, due to their potential stem character, another possible source of islet tissue are duct cells, which may be able to trans-differentiate to an endocrine fate (Slack, 1995). Thus, it is also possible that the excess of endocrine cells in *jagged2* morphants is at the expense of the forming duct. Alternatively, as exocrine cells differentiation takes place over a long time window, as judged by the slow and gradual appearance of *trypsin* expression, the precursor pool derived from the

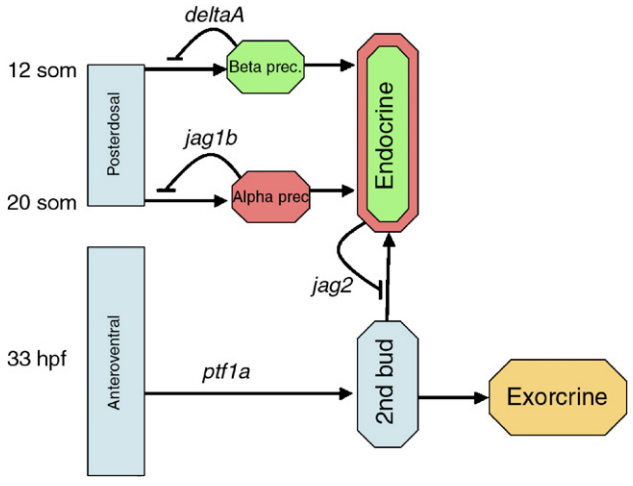

Fig. 9. Model describing the role of Notch-ligands in pancreatic cell differentiation. The timeline is on the left and relevant stages of pancreatic differentiation are indicated: beta-cells differentiating at 12-somite and alpha-cell differentiating at 20-somite stage will contribute to the first bud while the second bud, differentiating at 33 hpf, is supposed to form the exocrine tissue and some endocrine cells. The role of each Notch ligand in limiting the number of differentiating precursors is indicated.

anteroventral bud may have a propensity to endocrine differentiation if not maintained in the precursor state by *jagged2*. As there are no markers available to distinguish which lineage the surplus of *ins* expressing cells in *jagged2* morphant embryos derive from, we currently cannot distinguish these potential mechanisms.

The accelerated differentiation of the exocrine pancreas in *mib* mutants (Esni et al., 2004) together with our finding that the same embryos have an impaired final number of exocrine cells could be easily explained with depletion of pancreatic progenitors in the absence of Notch signalling. Therefore, our results are in agreement with a Notch-controlled disclosure of different pancreatic cell types from a common pool of progenitors, similar to what has been described in other species (Apelqvist et al., 1999; Murtaugh et al., 2003). This hypothesis was tested with our final set of experiments, using the heat-shock conditional N^{ICD} bi-genic system developed by Scheer and co-workers (Scheer and Campos-Ortega, 1999). Bi-genic embryos, heat shocked from specific time points to activate Notch signalling ubiquitously, lack all the pancreatic cell types that would normally differentiate after heat-shock, but exhibit all the cell types that differentiate before the shock. The persistence of the undifferentiated epithelium in N^{ICD} activated embryos, as revealed by *pdx1* expression, is consistent with a model of pancreas differentiation in which differentiating cells, by a ligand-specific Notch-mediated feedback mechanism, limit further differentiation of cells from the precursor pool. When N^{ICD} is expressed only in beta-cells, it leads to a depletion of beta-cells without any change in alpha- and delta-cell numbers. Two conclusions can be drawn from this finding. One could be that insulin-positive beta cells must be dividing to form more beta cells — as already shown in the adult mouse by Dor et al. (2004); unfortunately, we don't have evidence of enough beta-cell division to support the idea that the loss of beta-cells we observe in the zebrafish embryo could be mediated by N^{icd} inhibition of proliferation (F.A., W.D. unpublished results). Rather, the finding that activation of N^{ICD} in *insulin*-expressing cells does not increase the number of alpha- or delta-cells indicates that a) in vertebrate pancreas development Notch is not directly involved in the choice between different fates but in the maintenance of the precursor pool and b) N^{ICD} blocks beta-cells differentiation from committed precursors. Thus, the exit from the precursor state might require, even for the exocrine component, the cessation of Notch signaling in order to allow the cells to reach their final condition (Esni et al., 2004).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.09.041.

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